INTERACTIONS BETWEEN PLANT GROWTH PROMOTING MICROORGANISMS (PGPM) AND BIOCHAR

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ABSTRACT

Rhizobia are frequently used in the agriculture sector to enhance legume growth and improve soil fertility. There is growing interest in utilizing biological nitrogen fixation as a means of increasing the potential for sustainable intensification of food production whilst simultaneously reducing environmental damage caused by overuse of chemical fertilisers. Biochar, a recalcitrant carbon-rich product of pyrolysis which may be added to soil as a fertilizer or as a soil improver, alters soil physico-chemical properties usually by acting as a liming agent, by increasing water holding capacity or by modifying cation exchange capacity. The effects of biochar on the soil microbial community are not fully understood. Therefore, the main aim of this investigation was to evaluate the effects of biochar on the *Rhizobium*-legume relationship and determine whether biochar could increase legume growth. To achieve this aim, a series of growth experiments were carried out under controlled conditions in which broad bean (*Vicia faba*) was grown with *Rhizobium leguminosarum* and the symbiosis tested against three concentrations of biochar applied as a soil amendment and with two different char particle sizes. Beans responded well to *Rhizobium* under char-free conditions but the effects of biochar on the symbiosis were variable and depended on char particle size, concentration and *Rhizobium* strain (commercial or indigenous). Powdered char inhibited plant growth when in the presence of the commercial rhizobia, but not with indigenous strains. This is an important finding since commercial inocula are commonly used in agronomic situations. Plant available soil nutrients were modified by biochar and surprisingly by an interaction between char concentration and the two rhizobia strains. When beans were co-cropped with wheat, beans

performed better when grown with powdered char than without. This is in contrast to the response of bean plants to powdered char in the absence of any competition. Since wheat was generally the superior competitor, powdered char amendment enabled the bean to take advantage of the N-limiting environment that powdered char created and perform better than in the soils that advantaged the wheat. The investigation highlighted the complexity of the system, but identified the importance of char particle size and *Rhizobium* strain selection.

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1 Introduction

Energy, global food security and climate change are three issues that dominate current scientific thinking. Thermal gasification of agricultural wastes and residues will facilitate energy production and simultaneously produce char, which is a recalcitrant carbon-based material showing promise as a soil improver (Müller-Stover *et al.*, 2012) due to its ability to act as a liming agent, increase water holding capacity and modify cation exchange capacity (Warnock *et al.*, 2007; Hansen *et al.*, 2016). Furthermore, there is much interest in applying char to soils as a means of improving soil organic content and sequestering carbon because it may take thousands of years to degrade (Yang *et al.*, 2016). It is not surprising that there is much interest in producing and utilising char. When char is applied to soils, it is often referred to as biochar; both terms will be used interchangeably within this thesis.

Feedstock used and production conditions (e.g. pyrolysis, gasification, torrefaction) will dictate the quality of char produced and its suitability for soil application in the agronomic context (see Albuequerque *et al.*, 2016). Ideally, char properties could be modified for purpose, although guaranteeing good quality and consistent feedstock may be challenging. For example, changing pyrolysis temperature and retention time can alter pH due to removal of acid functional groups (Albuequerque *et al.*, 2016).

Plant growth improvements have been demonstrated following char amendment although data are variable (Jeffery *et al.*, 2011). The meta-analysis conducted by Jeffery *et al.* (2011) which considered findings across the globe, highlighted some interesting points; for example, biochar was most effective in medium and coarse textured soils, application rate does not make a difference to crop yield, it produces better results on acidic and neutral soils due to its liming effect and soybean and cowpea respond favourably to it. A key point also highlighted by these authors was that of biochar-related negative growth effects which have been reported in the literature.

Numerous reviews highlight the benefits of applying char to soils, yet there is an increasing awareness that there may be unintended consequences and further work is therefore required. Although char is recalcitrant it may still be degraded by soil microorganisms or undergo chemical oxidation, or physical breakdown (Cheng *et al.*, 2006).

Since according to Jeffery *et al.* (2011), soybean and cowpea respond favourably to char application, it is logical to study the potential benefits of applying char to legume crops in an attempt to enhance efficiency of the symbiosis. Legumes are a staple food for humans (FAO, 2016) and in addition to growing them in monocultures, they are also grown within agronomic situations that encompass intercropping, forage crops and cover cropping, all aimed at improving nutrient-use-efficiency (Duchene *et al.*, 2017).

Legumes fix atmospheric nitrogen and this makes them valuable in many agricultural settings where they may be intercropped or used as green manure as an alternative to chemical nitrogen fertilisers (Bedoussac *et al.*, 2015). Estimates of nitrogen fixed through biological nitrogen fixation (BNF) are in the range of 139 million to 175 million tonnes of nitrogen. Approximately 44 million tonnes of N are fixed from arable land and a further 45 million tonnes from pasture (Zahran, 1999).

Application of rhizobia strains to enhance legume yields is an important approach in sustainable agriculture (Stajković *et al.*, 2011) and there is growing interest in ways to increase nitrogen-use efficiency in arable systems and reduce reliance on chemical fertilisers which result in environmental degradation (Lu and Tian, 2017).

Some of the most important rhizobial species belong to the genus *Rhizobium* and the symbiosis between *Rhizobium leguminosarum* and broad bean (*Vicia faba*) is the mainstay of this thesis.

The rationale behind the work is predicated by a need to increase food production in a sustainable manner and simultaneously create a link within a food, energy and climate nexus.

The main objectives of the work were to:

- Determine the effects of biochar on the *Rhizobium*-legume symbiosis and establish whether there could be unintended consequences of using biochar in this context. Different char concentrations and particle sizes were tested. This objective was addressed in Chapter 2.

- Establish whether biochar modifies the competitive outcome between a legume and wheat. The aim was to simulate co-cropping. This objective was addressed in Chapter 3.

- Quantify whether biochar limits or enhances interactions within a tripartite system. In this case the partners were *Rhizobium leguminosarum*, *Vicia faba* and arbuscular mycorrhizal fungi. This objective was addressed in Chapter 4.

2 Interactions between biochar and *Rhizobium* during growth of broad bean (*Vicia faba*)

2.1 INTRODUCTION

Beans, including broad beans, are an important staple food crop for human consumption across the world and 2016 was designated International Year of Pulses (Food and Agriculture Organization of the United Nations, 2016). In nutritional terms, beans are an excellent protein source and are rich in minerals (especially iron and zinc) and also in vitamins (White and Broadley, 2009). Leguminous plants (including beans) can provide for their own nitrogen requirements through biological nitrogen fixation (BNF) in symbiosis with soil bacteria collectively known as rhizobia. These bacteria form root nodules on leguminous plants and convert atmospheric N_2 into ammonia which is subsequently assimilated into amino acids for biosynthesis. Application of effective rhizobial strains as biofertilizers to improve legume production is an important approach in sustainable agriculture (Stajković *et al.*, 2011). There is growing interest in ways to increase nitrogen-use efficiency in arable systems, partly because in many developing countries N-fertilisers are unaffordable and in modern conventional agricultural systems, most applied N-fertiliser is lost, resulting in environmental problems such as soil degradation, water eutrophication and air pollution (see Lu and Tian, 2017 and references therein).

Some of the most important rhizobial species belong to the genus *Rhizobium* and the symbiosis between *Rhizobium leguminosarum* and broad bean (*Vicia faba*) is the focus of this Chapter. Although able to fix N_2, legumes will preferentially take up soil N if available because of the carbon costs to the plant, usually resulting in a mixture of soil-derived and atmospheric nitrogen in the total N mass of the plant (Peoples *et al.*, 1995, 2009). Whilst fixation generally accounts for 50-70% of the total plant N, the amount of nitrogen fixed is related to the fitness of the plant (Bohlool *et al.*, 1992) and to environmental stressors such as drought (Marino *et al.*, 2007).

In addition to being cultivated as a staple food, legumes are often grown as cover crops in order to reduce nitrate losses from soils that would otherwise

remain bare over the winter and to simultaneously increase organic inputs into soils (Blesh, 2018). An alternative method of increasing soil carbon inputs and concurrently enhancing N retention is to amend soil with biochar. Biochar has been extensively studied with a view to increasing crop yield and soil fertility either directly or indirectly; for example, by modifying cation exchange capacity, pH or influencing water holding capacity (Lehmann and Joseph, 2009; Schomberg *et al.*, 2012). It has been suggested that biochar addition to soil can enhance soil fertility through increased BNF when legumes are present (Nishio, 1996; Rondon *et al.*, 2007), perhaps by enhancing the potential of the rhizobia-legume symbiosis (Kahindi *et al.*, 1997; Thies and Rillig, 2009) due to altered soil properties following biochar application (Rondon *et al.*, 2007). Biochar-related improved growth of soybean (*Glycine max*) (Tagoe *et al.*, 2008; Suppadit *et al.*, 2012) and of common bean (*Phaseolus vulgaris*) (Rondon *et al.*, 2007) under field and greenhouse conditions were reported; specifically, increased number of nodules, plant height, nutrient uptake, yield and dry weight. Nevertheless, there is a great deal of variation in published data for crop performance (across a range of crops) and the most likely consistent explanation for improved plant growth is a liming effect in acidic soils and increased water holding capacity (see Jeffery *et al.*, 2011 for a meta-analysis of published data). Compared to other crop species, limited attention has been paid to the influence of biochar on the important process of BNF, especially concerning the mechanisms of interaction between biochar and *Rhizobium* for improvement of growth and yield of leguminous plants.

Addition of biochar to soil will modify the soil C:N ratio. Some studies have demonstrated biochar-mediated increased nitrification rates (Nelissen *et al.*, 2012), whilst others reported immobilisation of soil N by char amendment (Bruun *et al.*, 2012). Any alteration in available N will potentially affect BNF and uptake of soil N by legumes and requires further study. Therefore, the **overall aim** of the experiment described in this Chapter was to determine whether soil amendment with char increased growth and nutrient uptake of broad bean when inoculated with a commercial rhizobia strain, or when left uninoculated. The experiment was a pot trial conducted under controlled conditions. Two char particles sizes were used, powdered char and 1 mm size particles. Two size particles were used because to date, particle size has largely been ignored, resulting in a knowledge gap that needs to be filled before char is applied on a

field-scale. However, Sasidharan *et al.* (2016) conducted a column experiment in which char of two sizes (>60 µm and 2 mm) was added to quartz and *Escherichia coli* cultures eluted through the column. The authors showed that recovery of *E. coli* was greater from the quartz column containing the larger char particles. Bacterial transport was thus lessened by the smaller char particles. Natural soils will be more complex than the quartz substrate used by Sasidharan *et al.* (2016); char-related changes in soil pH may influence bacterial retention by increasing electrostatic repulsion and therefore microbial transport within the soil. These interactions could affect microbial dynamics within the soil and effectiveness of the rhizobia.

The **specific objectives** of this experiment were:

(1) To determine if bean plant growth responds to soil char amendment.

(2) To quantify any growth effects resulting from addition of a commercial rhizobial inoculum.

(3) To establish whether char amendment affected the rhizobia-legume symbiosis.

The **main hypotheses** were:

(1) Addition of the commercial rhizobial inoculum would increase bean growth.

(2) Soil amendment with 1 mm size char would result in an additive growth effect beyond that of the rhizobial inoculum only, possibly due to increased soil fertility or to enhanced pH.

(3) Soil amendment with the powdered char would reduce rhizobial effectiveness due to bacterial retention and/or the powder 'clogging' soil pores.

(4) Soil amendment with a higher concentration of char would increase plant growth due to an indirect improvement in soil fertility through enhanced pH, or to a direct improvement in soil fertility.

2.2 MATERIALS AND METHODS

2.2.1 Experimental approach

A pot experiment was conducted in which a dwarf variety of broad bean (*Vicia faba*) was grown in soil, or in soil amended with one of two size fractions of char each at two concentrations. A commercial *Rhizobium* inoculum (*Rhizobium leguminosarum* biovar *viciae*) donated by Legume Technology (Nottingham, UK) was added to half the pots. The intention was to compare plants with and without the symbionts, grown with or without char under controlled conditions in a growth room.

2.2.2 Soil and char preparation

The char used for this investigation was purchased from the BioRegional HomeGrown Company Ltd. The char consisted of mechanically chipped trunks and large branches of *Fraxinus excelsior*, *Fagus sylvatica* and *Quercus robur* that had been pyrolysed at 450°C for 48h. The pH of the char was 9 and it contained negligible amounts of N.

Char was prepared by mashing and grinding lumps of charcoal and sieving to obtain a powder fraction (<0.7 mm) and a coarser fraction (1 mm). Sieved (2 mm) loam soil was mixed with Levington's Pot and Bedding Compost (2:1 soil:compost) and moisture content determined following oven drying subsamples of the soil/compost mixture at 105°C for 24h to enable calculation of the quantity of char to add on a dry weight equivalent basis. Soil/compost mixes (± char) were added to plastic plant pots (13.3 cm x 11.3 cm) prior to sowing seeds. The char concentrations used were calculated on a dry weight equivalent and each pot received either 7.5 g or 20 g of char, hereafter referred to as 2.5% and 7% char respectively. Char and soil/compost were thoroughly mixed. (Please note that the terms 'char' and 'biochar' are used interchangeably and mean the same thing.)

2.2.3 Experimental set up

The soil/compost mix was amended with char to give the following treatments:

(i) Soil/compost only (= control)

(ii) Soil/compost + 2.5% powdered char

(iii) Soil/compost + 7% powdered char

(iv) Soil/compost + 2.5% of 1 mm char

(v) Soil/compost + 7% of 1 mm char

Hereafter the soil/compost mix will be referred to as 'soil', or the 'no char' treatment. Eight pots of each char treatment were established, half of which received commercial *Rhizobium* inoculum and half remained uninoculated. This was applied by adding a 2 mL volume of inoculum to the planting hole prior to adding the bean seed. The inoculum carrier is peat and since the potting compost contained peat it was not necessary to add sterilised inoculum to the uninoculated pots. Two beans were sown in each pot and thinned after germination to give one plant per pot.

Therefore, each of the 5 treatments listed above (i-v) was duplicated to give one set with added *Rhizobium* and one set without added *Rhizobium*. There were 4 replicate pots per treatment.

The pots were placed in a growth room with 12 hours light-dark cycle with a daytime temperature of 20°C and a night-time temperature of 18°C. The plants were watered daily as required with deionized water. The experimental design was a fully factorial randomised block design with 4 replicate blocks. Plants were maintained to the flowering stage when they were harvested.

2.2.4 Harvest and sample processing

At the flowering stage, plants were carefully removed from the pots and roots and shoots separated. Roots were rinsed in tap water and blotted dry. Three root lengths were randomly selected from each root system and root length measured and number of nodules counted. These root portions were then oven dried (45^{o}C until consistent weight) so that the number of nodules could be expressed on a cm^{-1}, a g^{-1} and a per whole root system basis. Thirty nodules were also randomly selected and removed from the remaining root system and oven dried. The rest of the root system was also oven dried and the various dry weights combined later to give the whole root biomass. It should be noted that

uninoculated plants were also nodulated, so this process was carried out for all plants. Shoots were also oven dried at 45°C until constant weight was reached.

Soil from each pot was homogenised and subsamples taken for chemical analyses that required fresh soil, whilst the remaining soil was oven dried at 105°C.

2.2.4.1 Exchangeable and available soil elements in fresh soil

Exchangeable and available macro- and micro-nutrients in the soil were determined following extraction in 1 mol L^{-1} ammonium nitrate (NH_4NO_3) and in Milli-Q water respectively. For both analyses, 2 g fresh soil (sieved and homogenised) were weighed into 50 mL centrifuge tubes. Eighteen mL of the appropriate extractant were added and the tubes laid horizontally on an orbital shaker (KS 500 Janke & Kunkel IKA Labortechnik) for one hour at 256-259 rpm and then centrifuged (Herml Z400) at 3000 rpm for 20 minutes. The extracts were then filtered (through Whatman No. 42 filter paper) into 30 mL Universal tubes and subsequently diluted 1:10 with 2% nitric acid (HNO_3) (1 mL sample:9 mL diluent). Following dilution, elemental concentrations were determined using inductively coupled plasma mass spectrometry (ICP-MS; Thermo-Fisher Scientific X-Series^{II}, Thermo Fisher Scientific Inc., Waltham, MA, USA). Data are only shown for the water extracts because anomalies were observed throughout data sets following ammonium nitrate extractions. The reason for this is unknown, but might be because the NH_4NO_3 concentration was insufficient for optimal elemental exchange in the presence of powdered char.

2.2.4.2 Extractable nitrogen (TN) and organic carbon (TOC) in fresh soil

Extractable nitrogen and organic carbon in the soils were determined following potassium sulphate (0.5 mol L^{-1} K_2SO_4) extraction. Two g of fresh soil (well-mixed) from each pot were weighed into 50 mL centrifuge tubes. Ten mL of K_2SO_4 solution were added to the soil and tubes were shaken on an orbital shaker and centrifuged as described above (section 2.2.4.1). The extracts were then filtered as above and diluted with Milli-Q water (1:10) prior to